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The paracellular permeability of opossum kidney cells, a proximal tubule cell line

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Background. The regulation of the unusually leaky paracellular pathway of the proximal tubule is poorly understood partially because of the lack of an appropriate *in vitro* cell model. In this study, we determined whether the paracellular permeability of opossum kidney (OK) cells would resemble that of the *in vivo* proximal tubule epithelium.

Methods. The parental and subclonal OK cells and, for comparison, LLC-PK_1 cells were cultured on permeable Transwell supports. The apparent paracellular permeability coefficient (P_{app}) for the extracellular marker ^{3}H-mannitol was determined.

Results. The P_{app} of OK cell sheets (12.17×10^{-6} cm/sec) was remarkably close to the previously reported P_{app} of rat proximal tubules. The P_{app} of LLC-PK_1 cells, another proximal tubule cell line, however, was approximately 20-fold lower than that of both OK cells and the *in vivo* proximal tubule. Phorbol 12-myristate 13-acetate, a protein kinase C activator, enhanced the P_{app} of OK cell sheets. The characteristic response of paracellular permeability to Ca^{2+} switch was demonstrated in OK cell sheets. Slight variations of P_{app} among several OK subclones were observed. Basal to apical P_{app} was uniformly higher than apical to basal P_{app}, independent of cell subtype. This rectification was attenuated by inhibition of active transport.

Conclusions. OK cell sheets cultured on Transwell supports possess a leaky paracellular pathway resembling that of the proximal tubule epithelium *in vivo.*

The proximal tubule reabsorbs approximately 60% of the fluid and a large portion of the solutes filtered by the glomeruli. In addition to the much studied transcellular pathway, a substantial amount of fluid and solute can also be transported via the paracellular pathway [1]. Previous *in vivo* studies in our and other investigators' laboratories have demonstrated that the paracellular transport in the proximal tubule is under dynamic regulation [2] and may be directly involved in the functional changes seen in diseases such as acute renal failure [3]. The understanding of proximal tubule paracellular permeability, however, has been rather limited [1], partially because of the lack of an appropriate *in vitro* cell model.

The paracellular pathway of the proximal tubule shares some common characteristics with other epithelia, such as being gated by tight junctions. On the other hand, it also possesses unique properties distinct from other epithelia, especially from other nephron segments. One of the most striking characteristics of the paracellular pathway of the proximal tubule is its unusual leakiness. The transepithelial electrical resistance (TER), a measurement inversely correlating with the paracellular permeability, of the *in vivo* proximal tubule was reported to be 5.6 to 11.6 Ωcm^2 in several different species [4–6], whereas the TER of the distal tubule is two orders of magnitude higher [7]. An *in vitro* cell model could be appropriate for studying the paracellular permeability of the proximal tubule only if it possesses a similarly leaky paracellular pathway.

In this study, we investigated and compared two established proximal tubule cell lines, opossum kidney (OK) cells and LLC-PK_1 cells, in terms of paracellular permeability. We found that the paracellular permeability of OK cells, but not LLC-PK_1, remarkably resembled that of the *in vivo* proximal tubule. Several important properties of the paracellular permeability of OK cells were also characterized.

METHODS

Cell culture

Opossum kidney and LLC-PK_1 cells were originally obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). OK subclones were kindly provided by Dr. Leonard Forte (University of Missouri). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and were incubated in 5% CO_2/95% air at

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37°C. Cells were subcultured when they reached confluence by incubation with 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA). For paracellular transport studies, confluent cells were trypsinized and seeded on the inserts (0.4 μm pore size, 1 cm^2 growth area) of the permeable Transwell supports.

Apparent paracellular permeability coefficient measurement

The paracellular permeability coefficient (P_{app}) for D-mannitol, a hydrophilic and neutral extracellular fluid marker, was measured in a transport medium containing (in mM) NaCl 112, KCl 4.5, NaH_2PO_4 1, $NaHCO_3$ 25, $CaCl_2$ 1.8, $MgSO_4$ 0.5, D-glucose 5, and HEPES 10 (pH 7.4). When the P_{app} was measured in Na^+-free medium, NaCl, NaH_2PO_4, and $NaHCO_3$ in transport medium were equimolarly replaced with choline chloride, KH_2PO_4, and choline bicarbonate, respectively. One μCi/ml of ^{3}H-mannitol was included in the donor chamber. After incubation for 5 to 30 minutes, depending on the experimental conditions, duplicate samples were taken from the receiver chamber, and the radioactivity was counted using a scintillation counter (Beckman LS 6000SC). The P_{app} was then calculated as P_{app} (cm/sec) = $[C_R/C_D/t(min)] \times V_R(ml)/[A(cm^2) \times 60]$, where C_R was the final ^{3}H-mannitol concentration in the receiver chamber, C_D was the initial ^{3}H-mannitol concentration in the donor chamber, t was the incubation time, V_B was the volume of transport medium in the basal chamber, and A was the surface area of cell sheet, which was 1 cm^2 in our studies.

Na^+ uptake assay

Na^+ uptake assay was performed as described [8] with minor modifications. NH_4^+-loading medium contained (in mM) choline chloride 80, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, D-glucose 5, HEPES 25, and NH_4Cl 50 [pH adjusted to 7.4 with Tris (hydroxymethyl) amino methane]. Na^+ uptake medium contained (in mM) choline chloride 130, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, D-glucose 5, HEPES 25, NaCl 15, pH 7.4. One mM ouabain was included in the basal chamber, whereas 0.2 μCi/ml ^{22}Na with or without 10 μM 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) was included in the apical chamber. After stopping the uptake, the cell sheets were cut out together with the permeable support and counted with a scintillation counter.

Materials

^{3}H-mannitol and cell culture medium were from Sigma Chemical (St. Louis, MO, USA). ^{22}Na was from NEN Life Science (Boston, MA, USA). Transwells and other plastic cell culture supplies were from Corning Costar (Cambridge, MA, USA).

Statistics

Data are shown as means ± SEM. The *N* values shown represent the number of separate cell sheets that were divided into several experiments with each one done in duplicate or triplicate. Paired *t*-test, unpaired *t*-test, or one-way analysis of variance followed by the Student–Newman–Keuls test were used when appropriate. A *P* value of less than 0.05 was considered statistically significant.

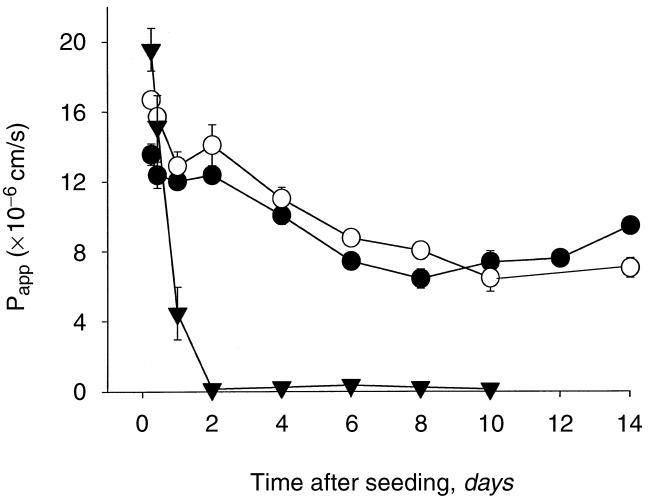

Fig. 1. Changes of paracellular permeability after seeding. Cells were seeded on permeable Transwell supports at the indicated densities, and the paracellular permeability coefficient (P_{app}) for ^{3}H-mannitol was measured at 6, 10, 24, and 48 hours and then every other day after seeding. The measurements were performed at 22°C (*N* = at least 4 for each time point). Symbols are: (●) OK, $5.0 \times 10^5/cm^2$; (○) OK, $2.5 \times 10^5/cm^2$; (▼) LLC-PK_1, $2.5 \times 10^5/cm^2$.

RESULTS

Paracellular permeability coefficient of OK and LLC-PK_1 cells and its change with time after seeding

As shown in Figure 1, when OK cells were seeded on permeable supports at a density of $2.5 \times 10^5/cm^2$ and the P_{app} for ^{3}H-mannitol was measured in the apical to basal direction at room temperature (22°C), the P_{app} decreased with time, reached $7\sim8 \times 10^{-6}$ cm/sec four to six days after seeding and remained at this level. At this time, the P_{app} of OK cell sheets was $12.17 \pm 0.86 \times 10^{-6}$ cm/sec when measured at 37°C (*N* = 6). When the seeding density was increased to $5.0 \times 10^5/cm^2$, the change of P_{app} with time followed a similar pattern. When the P_{app} of LLC-PK_1 cells seeded at $2.5 \times 10^5/cm^2$ was measured, a much sharper drop of P_{app} was observed during the first two days after seeding. The stable level of the P_{app} for ^{3}H-mannitol of LLC-PK_1 cells was approximately 0.2×10^{-6} cm/sec at 22°C and was $0.47 \pm 0.06 \times 10^{-6}$ cm/sec (*N* = 6) at 37°C. Because the P_{app} of OK cells appeared to be much closer to that measured in *in vivo* proximal tubules, we focused more on OK cells while

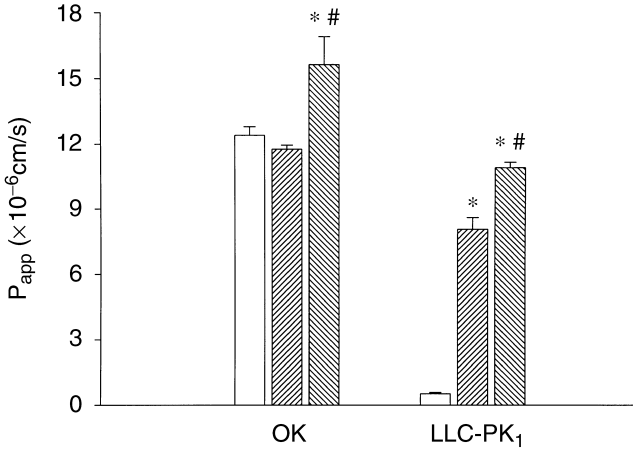

Fig. 2. Effect of phorbol 12-myristate 13-acetate (PMA) on the P_{app} of OK and LLC-PK$_1$ cells. Cells on Transwell supports were incubated in the regular cell culture incubator with culture medium containing 0.1 μM PMA for the indicated time before the P_{app} for ^{3}H-mannitol was measured at 37°C. Symbols are: (□) control; (▨) PMA 0.1 μm, 1.5 hr; (▧) PMA 0.1 μM, 5 hr; *$P < 0.05$ vs. control; #$P < 0.05$ vs. 1.5 hours ($N = 5$ for OK cells and 4 for LLC-PK$_1$ cells).

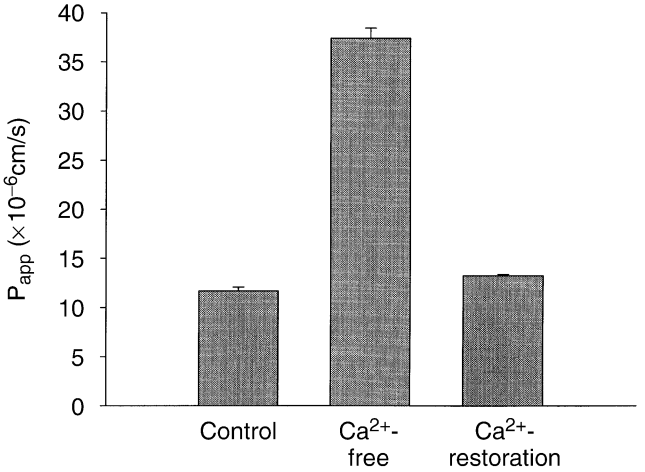

Fig. 3. Response of the P_{app} of OK cell sheets to Ca^{2+} switch. Ca^{2+} switch was performed as described in the **Methods** section. The P_{app} for ^{3}H-mannitol was measured at 37°C. Ca^{2+} free = PBSA/1 mM EDTA for one hour; Ca^{2+} restoration = returned to transport medium (containing 1.8 mM Ca^{2+}) for five hours ($N = 4$ to 6).

using LLC-PK$_1$ cells as a comparison when appropriate. In the following studies, cells were used when the P_{app} reached the stable level.

In spite of the relatively high permeability, the OK cell sheets were intact as indicated by measuring the movement of horseradish peroxidase (HRP) across the cell sheets. Fifty μg/ml HRP were included in the culture medium in the basal chamber. After six hours of incubation, only 0.58 ± 0.20% ($N = 5$) of HRP appeared in the apical chamber. HRP was measured as described [9] with minor modifications.

Effect of phorbol 12-myristate 13-acetate on the P_{app}

In a series of previous studies, activation of protein kinase C (PKC) was found to enhance dramatically the paracellular permeability of LLC-PK$_1$ cell sheets [10–12]. As depicted in Figure 2, incubation with 0.1 μM phorbol 12-myristate 13-acetate (PMA) for 1.5 hours did substantially increase the P_{app} of LLC-PK$_1$ cell sheets 17-fold from 0.52 ± 0.07 × 10^{-6} cm/sec to 8.06 ± 0.54 × 10^{-6} cm/sec ($N = 4$, $P < 0.05$). Increasing the incubation time to five hours further enhanced the P_{app} to 10.90 ± 0.26 × 10^{-6} cm/sec ($N = 4$, $P < 0.05$ vs. control or 1.5 hr). Incubation of OK cells with PMA at the same concentration for 1.5 hours did not change the P_{app} (12.40 ± 0.39 × 10^{-6} cm/sec vs. 11.76 ± 0.19 × 10^{-6} cm/sec, $N = 5$, $P > 0.05$). When the incubation time was increased to five hours, the P_{app} of OK cells was significantly increased to 15.63 ± 1.27 × 10^{-6} cm/sec ($N = 5$, $P < 0.05$ vs. control or 1.5 hr).

Response of the P_{app} of OK cells to Ca^{2+} switch

It is well-established that a normal extracellular Ca^{2+} concentration is required to maintain the tight junction structure. Indeed, incubating the OK cell sheets in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (PBSA) with 1 mM EDTA for one hour (Ca^{2+}-free) caused the P_{app} of OK cell sheets to increase from 11.67 ± 0.41 × 10^{-6} cm/sec to 37.42 ± 1.05 × 10^{-6} cm/sec ($N = 4$, $P < 0.001$; Fig. 3). Under the light microscope, OK cells after this maneuver appeared to round up and separate from each other, although they remained attached to the permeable supports. After replacing the Ca^{2+}-free medium with transport medium containing 1.8 mM Ca^{2+} for five hours (Ca^{2+} restoration), the P_{app} recovered to a value close to the control (Fig. 3).

Table 1. Bi-directional P_{app} ($\times 10^{-6}$ cm/sec) of opossum kidney (OK) cell and OK sub-clones cultured on permeable supports ($N = 8$)

	Apical to basal	Basal to apical
OK	14.78 ± 0.43[b]	20.71 ± 0.84[a]
OK-E	10.73 ± 0.53	19.12 ± 1.25[a]
OK-B	9.98 ± 0.46	16.37 ± 0.72[a]
OK-P	12.56 ± 0.37[c]	23.06 ± 2.00[a]
OK-H	9.05 ± 0.51	18.34 ± 1.08[a]
Supports	46.17 ± 1.83	55.45 ± 0.91[a]

[a] $P < 0.05$ vs. apical to basal
[b] $P < 0.05$ vs. OK-E, OK-B, OK-P, and OK-H
[c] $P < 0.05$ vs. OK, OK-E, OK-B, and OK-H

Opossum kidney cell sub-clones

Several OK sub-clones with higher morphology homogeneity have been established [13]. The P_{app} of parental OK cells and several OK sub-clones cultured on Transwell supports is in the same range and all close to that of proximal tubule *in vivo*, although statistically significant variations of P_{app} among different subtypes were observed (Table 1).

Table 2. Effects of Na^+-removal, temperature, and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) on the P_{app} ($\times 10^{-6}$ cm/sec) of OK cells ($N = 6$)

	Apical to basal	Basal to apical	Δ^b
Control	12.17 ± 0.86	20.20 ± 1.09	8.03 ± 1.61
Na^+-free	13.61 ± 0.59	15.67 ± 0.51[a]	2.06 ± 0.74[a]
22°C	9.89 ± 0.64	14.08 ± 1.52[a]	4.19 ± 1.05[a]
10 μM EIPA	13.51 ± 1.32	19.55 ± 0.90	6.04 ± 1.81

[a] $P < 0.05$ vs. control
[b] Δ = (basal to apical) – (apical to basal)

Table 3. Opossum kidney (OK), but not LLC-PK_1, cell sheets possess a leaky paracellular pathway resembling that of the *in vivo* proximal tubule

	P_{app} for mannitol $\times 10^{-6}$ *cm/sec*	TER Ωcm^2
In vivo proximal tubule (rat)	8.70 [15[a]]	5.6~11.6 [7]
OK cell sheets	12.17	5~10+ [16]
LLC-PK_1 cell sheets	0.47	100~200 [17]

Abbreviations are: P_{app}, apparent paracellular permeability coefficient; TER, transepithelial electrical resistance.
[a] see References

Bidirectional measurements of P_{app} and the effect of transcellular transport inhibitions

As shown in Table 1, for all OK cell subtypes tested, the basal to apical P_{app} was uniformly higher than apical to basal P_{app}. Inhibition of transcellular transport by either removing Na^+ from transport medium (Na^+-free), lowering temperature to 22°C, or the presence of 10 μM EIPA, a Na^+/H^+ exchanger inhibitor, did not significantly alter the unidirectional apical to basal P_{app}, although a tendency for changes appeared to exist. Ten μM EIPA decreased Na^+ uptake from 4.59 ± 0.49 nmol/min/cm^2 to 0.96 ± 0.34 nmol/min/cm^2 ($N = 8$, $P < 0.05$). Importantly, these maneuvers significantly attenuated or tended to attenuate the difference between bidirectional measurements (Table 2).

DISCUSSION

The results of this study demonstrate that the P_{app} for the extracellular marker, D-mannitol, of OK cell sheets cultured on permeable Transwell supports were remarkably similar to the reported P_{app} value for mannitol in the *in vivo* rat proximal tubule (12.17×10^{-6} cm/sec in OK cells vs. 8.7×10^{-6} cm/sec in the *in vivo* rat proximal tubule) [14]. Alternatively, the P_{app} of the LLC-PK_1 proximal tubular cell line was approximately 20-fold lower than both the *in vivo* proximal tubule and the OK cells. These data of P_{app} are also in good agreement with the previous TER measurements showing that the TER of OK cells [15] was remarkably similar to the TER reported for the *in vivo* proximal tubule [4–6], whereas the TER of LLC-PK_1 cells was much higher [16] (Table 3).

The OK cell line has been used extensively for the study of phosphate transport due to its responsiveness to parathyroid hormone (PTH) [17]. In addition to PTH-sensitive phosphate transport, OK cells also mimic the renal proximal tubular epithelial cells in a variety of ways [18], including the formation of tight junctions [17]. When cultured on permeable supports, OK cell sheets have been shown to have polarized phosphate transport [19, 20] and polarized distributions of Na^+/H^+ exchangers [21] and Na^+,K^+-ATPase [19]. Furthermore, as shown in this study, the paracellular permeability of OK cell sheets increased in response to a Ca^{2+}-free medium and recovered in a medium containing a normal concentration of Ca^{2+}, which confirms that the intercellular junctions of OK cells share the common response of epithelial intercellular junctions to Ca^{2+} switch.

Phorbol 12-myristate 13-acetate (PMA), which has been shown to increase the paracellular permeability of LLC-PK_1 cells through activation of PKC_α [12], also increased the P_{app} of OK cell sheets. Compared with LLC-PK_1 cells, however, a longer incubation time was required to observe this effect. This might be due to the higher basal P_{app} level in OK cells compared with LLC-PK_1 cells or the existence of intrinsic differences between the regulation of tight junctions in OK and LLC-PK_1 cells.

The P_{app} of parental OK cells and various OK subclones is all close to that of the proximal tubule *in vivo* and much higher than that of LLC-PK_1 cells. The differences among OK sublines are slight, but significant. This may be caused by different degrees of homogeneity or other intrinsic properties of these sub-clones.

Another significant observation in this study is the rectification of P_{app}. Basal to apical P_{app} was consistently higher than apical to basal P_{app} in all OK cell lines tested. Although the basal to apical P_{app} of supports alone was also higher than the apical to basal P_{app}, two observations strongly indicate that the rectification of P_{app} observed in the presence of cell sheets was at least partially intrinsic to cell sheets *per se.* First, the basal to apical P_{app} of supports was approximately 20% higher than the apical to basal P_{app}, whereas the difference ranges from 40% to more than 100% in the presence of cell sheets. Second, inhibition of transcellular transport attenuated this rectification.

Simple diffusion, as a component of paracellular solute flux, will result in equal bidirectional apparent permeability. However, in addition, water flux through the paracellular pathway could alter solute flux through the solvent drag mechanism [22, 23]. In an *in vivo* microperfusion study performed in rat proximal tubules, Whittembury et al found that the major portion of paracellular solute flux was due to solvent drag rather than diffusion, and the paracellular solute flux was a function

of fluid reabsorption [23]. This is in agreement with our data showing that inhibition of transcellular transport attenuated the rectification of paracellular flux. We speculate that under the conditions of our *in vitro* experiments, fluid accumulates in the basal chamber because of the transcellular transport and the lack of removing mechanisms that are present *in vivo.* As a result, significant water backleak from the basal to apical compartment occurs, which could "drag" solutes with it and make the apparent basal to apical permeability for those solutes appear to be higher than the apical to basal permeability. This could explain the rectification of P_{app} and the attenuating effect of transcellular transport inhibition on this rectification observed in this study. The lack of P_{app} rectification in some previous cell culture studies might be due to the negligible paracellular fluid flux in those preparations. Therefore, this study favors the notion that paracellular solute flux can be more complicated than a simple diffusion process and may involve solvent drag effects as well.

In conclusion, OK cell sheets cultured on Transwell supports possess a leaky paracellular pathway resembling that of the proximal tubule epithelium *in vivo.* The results of this study, together with the abundant literature showing other aspects of the similarity between OK cells and the proximal tubule epithelium, strongly suggest that OK cells cultured on permeable Transwell supports may serve as a useful *in vitro* tool to study the regulation of paracellular permeability of the renal proximal tubule.

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